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The combination of electric current and copper promotes neuronal differentiation of adipose-derived stem cells

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Abbreviated title for the running head: Neuronal differentiation of ADSCs

Abstract and key terms

Damage to the nervous system can be caused by several types of insults, and it always has a great effect on the life of an individual. Due to the limited availability of neural transplants, alternative approaches for neural regeneration must be developed. Stem cells have a great potential to support neuronal regeneration. Human adipose-derived stem cells (hADSCs) have gained increasing interest in the fields of regenerative medicine due to their multilineage potential and easy harvest compared to other stem cells. In this study, we present a growth factor-free method for the differentiation of hADSCs toward neuron-like cells. We investigated the effect of electric current and copper on neuronal differentiation. We analyzed the morphological changes, the mRNA and protein expression levels in the stimulated cells and showed that the combination of current and copper induces stem cell differentiation toward the neuronal lineage with elongation of the cells and the upregulation of neuron-specific genes and proteins. The induction of the neuronal differentiation of hADSCs by electric field and copper may offer a novel approach for stem cell differentiation and may be a useful tool for safe stem cell-based therapeutic applications.

Key terms: Adipose-derived stem cell, Electric current, Copper, Differentiation, Neurons

Introduction

Due to aging, diseases and injuries to the nervous system, and the limited medical treatment options currently available, there is a clear need for induced neuronal regeneration. Embryonic, adult, and induced pluripotent stem cells have been intensively studied for the regeneration of neural tissues. Several adult stem cells sources can give rise to neurons including; bone marrow stromal cells[35], skin[21], dental stem cells[46], and adipose-derived stem cells[34]. Issues related to the use of embryonic and induced pluripotent stem cells include the ethical issues and formation of teratomas and immunogenicity *in vivo* [9,12], and these complications could potentially be overcome through the use of adipose-derived stem cells (ADSC). Adipose tissue is one of the most accessible sources for stem cells with high proliferative capacity and multilineage potential [8,32]. Pluripotent ADSCs are currently investigated as a promising cell source for clinical application, and they have the capability of neuronal differentiation [3,20,34].

The main methods currently used for differentiating ADSCs toward neurons are genetic manipulation, the promotion of neurosphere formation, and the use of different cytokines, growth factors or chemical reagents [3,7,16]. Although the latter is the most common method for neural differentiation, there are many issues associated with the use of growth factors and chemical reagents. Each factor must be critically reviewed by the relevant authorities before its use in translational studies. In addition, some reagents currently used for neurogenic differentiation, including dimethylsulfoxide (DMSO), β-mercaptoethanol (BME) and butylated hydroxyanisole (BHA) are criticized due to cell toxicity and induced cell stress [23,29].

Endogenous electric fields play an important role in many physiological processes, including cell migration, proliferation and differentiation, as well as wound healing [14,26]. Furthermore, external electric fields have been shown to cause cell orientation and elongation [10,40]. In stem cells, elongation and other morphological changes are often related to differentiation [34]. It has been shown that the electric field stimulation of neuronal pre-differentiated embryonic stem cells remarkably increases their differentiation [45]. In addition, Matos et al. reported the different effects of alternating electric fields, which were applied through nickel electrodes, on neural stem cell viability and differentiation [25]. They showed that neuronal differentiation was either enhanced or suppressed depending on the electric field frequency and the exposure time. Recently it has also been shown that human mesenchymal stem cells (hMSC) have a capability to differentiate into neuron-like cells when cultured on conductive substrate under electric fields [42]. These findings suggest that the use of an applied electric field is a potential tool to influence neuronal differentiation of stem cells.

In addition to electric field, we propose that another stimulus type for triggering and maintaining the neuronal differentiation is needed. In the central nervous system, a high concentration of copper is found, and reduced concentrations can be related to several neurological disorders [15,44]. Neuronal copper metabolism relies on extracellular copper sources and is mediated by copper-containing proteins [31,33]. It has been shown that copper is needed for the neurite outgrowth mediated by nerve growth factor signal transduction [5]. Copper is also found to modulate the osteogenic and adipogenic differentiation of mesenchymal stem cells [32]. Presence of copper may be important already at the early stages of stem cell differentiation as it may take part in both the commitment and maturation steps of the differentiation process. [32] Copper, that binds with high affinity to phosphatidylserine (PS), phospholipid enriched especially in neuronal membranes [27], may be needed to initiate the ADSC

differentiation towards neuronal lineage. In addition, mammalian copper transporter Ctr1 that has been found at plasma membranes and that is responsible of copper transport into the cell, has been suggested to be important in signal transduction mechanism involved in stem cell differentiation [11]

In this study, we investigated the possibilities of using the combination of electric fields and copper to promote the neuronal differentiation of adult stem cells. Our overall aim was to develop a growth-factor-free method for differentiation of ADSCs toward the neuronal lineage for clinical translation.

Materials and Methods

Isolation, expansion, and characterization of ADSCs in vitro

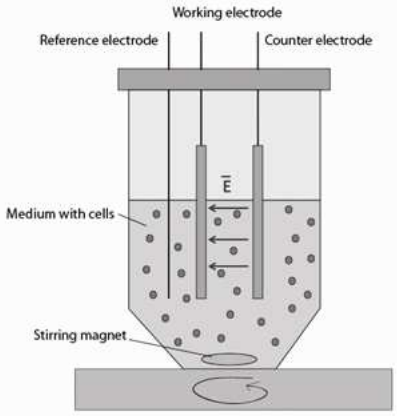
The ADSCs were isolated from adipose tissue samples collected from the subcutis/pelvic region or breast of female patients (n = 3, age = 52 ± 12 years) undergoing elective surgical procedures in the Department of Plastic Surgery at Tampere University Hospital (Tampere, Finland). The human ADSCs were isolated and characterized at passage 5-6 by FACS using lineage-specific markers as described previously [22]. Shortly, the adipose tissue was minced manually into small fragments and digested with 1.5 mg/mL collagenase type I (Life technologies, Paisley, UK). The digested tissue was centrifuged and filtered to separate the ADSC from the surrounding tissue. The isolated cells were then expanded in Dulbecco`s modified Eagle medium (DMEM/F-12 1:1) supplemented with 1% Glutamax I, 1% antibiotics/antimycotic and serum from 10% FBS, all purchased from Life technologies, Paisley, UK. Cultured ADSCs at passages 3-5 (n=4) were analyzed with monoclonal antibodies with flow cytometry (FACSAria; BD Biosciences, Erembodegem,

Belgium). Monoclonal antibodies against CD14-PE-Cy7, CD19-PE-Cy7, CD45RO-APC, CD49D-PE, CD73-PE, CD90-APC, CD106-PE-Cy5 (BD Biosciences Pharmingen); CD34-APC, HLA-ABC-PE, HLA-DR-PE (Immunotools GmbH Friesoythe, Germany); and CD105-PE (R&D Systems Inc, MN, USA) were used. Analysis was performed on 10000 cells per sample, and the positive expression was defined as the level of fluorescence 99 % greater than the corresponding unstained cell sample.

Experimental design

ADSCs were cultured to passage 5 or 6 in DMEM/F-12 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% glutamax and 1% penicillin-streptomycin (all from Life technologies, Zug, Switzerland). Prior to the stimulation, cells were trypsinized and seeded as a suspension in a 50-ml falcon tube with a total volume of 10 ml of culture medium and cell number of 1.5 to 2 million cells with the cell viability of 93 – 95 %. ADSCs were magnetically stirred in the tube, and in addition to the stirring the cells were subjected to electric current and/or copper for one hour. The stimulation groups and parameters are presented in figure 1. Shortly, ADSCs were stimulated with two different current densities with corresponding copper concentrations and with copper or current alone.

Figure 1. Experiment setup with the different experimental conditions. ADSCs were stimulated for one hour with copper, current or both. Copper was released to the cell suspension either abruptly or gradually via electrolysis. Current used was 1 mA or 1.5 mA applied through copper electrodes or 1 mA applied through platinum electrodes. The direction of the electric field is marked with arrows.



Stimulation group	Current	Copper
Control	no	no
Copper	no	24 µg/ml, abrupt
1 mA	1 mA	no
Copper + 1 mA	1 mA	24 µg/ml, gradual
Copper + 1.5 mA	1.5 mA	36 µg/ml, gradual

Figure 1. Experiment setup with the different experimental conditions. ADSCs were stimulated for 1 h with copper, current or both. Copper was released to the cell suspension either abruptly or gradually via electrolysis. Current used was 1 mA or 1.5 mA applied through copper-containing electrodes or 1 mA applied through pure platinum electrodes. The direction of the electric field is marked with arrows.

A three-electrode system was used for the application of the current. The working and counter electrodes were stainless steel electrodes (G Popp & Co AG, Zurich, Switzerland) that were further modified by C. Jentner Oberflächen- und Galvanotechnik (Pforzheim, Germany). The electrodes were first coated with copper and then with a very thin layer (approximately 0.2 µm) of palladium to enable their coating with platinum (thickness of 1 µm) on the uppermost layer of the electrode. The electrodes were then cut to the desired size, and thus the copper coating was exposed. Prior to the stimulation, all the electrodes were disinfected in 70% ethanol and rinsed with phosphate buffered saline (PBS).

Due to the current applied, copper (Cu(II)) was released gradually to the cells via electrolysis. The copper mass values (µg/ml) presented in the Fig. 1 are calculated by using Faraday`s laws of electrolysis m = (Q/F) * (M/z) where m is the mass of the

released copper, Q is the total electric charge passed through the electrode with current of 1 mA or 1.5 mA, M is the molar mass of copper and z is the electrons transferred per ion. In addition, the mass of released copper was determined by weighing the electrodes before and after applying the current. The electrodes were rinsed once with milliQ water and dried with N_2 before weight measurements.

A silver wire was used as a reference electrode. When the cells were stimulated with current of 1 mA without copper, working and counter electrodes made of 99.9% pure platinum were used. For all of the stimulations with a current, a monophasic pulsed current (current 5 seconds on, 20 seconds off) was applied for one hour with a PG580 potentiostat/galvanostat (Princeton Applied Research, TN, USA) in galvanostatic mode. The working and counter electrode were parallel to each other with a distance of 10 mm. The electric field E = U/d was 35 mV/mm in Cu + 1 mA, 53 mV/mm in Cu + 1.5 mA, and 155 mV/mm in 1 mA stimulation groups.

For the stimulation with the copper alone, copper was first released into a medium from the copper electrode via electrolysis with 1 mA in the absence of cells, and the copper-containing medium was then collected and added to the cell suspension all at once (adrupt release). Cell suspension was further mechanically stirred without current present for one hour.

After the stimulation, cells and the medium were collected and 4 000, 3 000 or 2 000 cells were seeded in chamber slides for 4 day, 7 day, and 14 day immunohistochemical analysis, respectively. Rest of the cells were seeded in culture flasks (real-time PCR and western blotting) and cultured for 4, 7, or 14 days as well. At day 3, medium still containing copper from the stimulation, was exchanged with the control culture medium, namely DMEM/F-12 supplemented with 20% heat-inactivated fetal bovine serum (FBS), 1% glutamax and 1% penicillin-streptomycin in

all experimental conditions. Later, medium was changed every three days. All of the experiments were performed in triplicates.

Cell viability and proliferation

Cell number and viability before and right after the stimulation, before the cells were plated into the chamber slides or culture flasks, were determined using a Countess® automated cell counter (Life Technologies, Zug, Switzerland). Cell proliferation rate on chamber slides during the 14 d culture time was determined by counting the dapi stained cells at day 4, 7, and 14. The cell proliferation is presented as relative values compared to day 4 control.

Real-time PCR

The total RNA was isolated using the SV Total RNA Isolation System kit (Promega, Dübendorf, Switzerland) according to the manufacturer's protocol, which included DNase digestion. The RNA was reverse transcribed with random primers (High-Capacity cDNA Reverse Transcription, Applied Biosystems). Pre-designed primers for rat Beta III-tubulin (HS00964962-g), MAP-2 (Hs01110346-m1), and eukaryotic 18S rRNA endogenous control (VIC®/MGB Probe, Primer Limited) were purchased from Applied Biosystems. The data were quantitatively normalized with the expression of 18S and were analyzed by measuring the threshold cycle (CT) values. For the quantification, the expression of each gene in the ADSCs was considered the 100% reference value. All of the values for mRNA expression were compared with the undifferentiated control.

Immunofluorescent staining

The ADSCs were cultured on Lab-Tek 4-well chamber slides (Thermo Scientific,

Nunc, Fisher Scientific AG, Wohlen, Switzerland) in growth medium for 4, 7, and 14 days after the stimulation. The cells were fixed in 4 % paraformaldehyde and permeabilized with 0.5% Triton X-100. The indirect immunostainings were performed at 4°C overnight. The neurogenic differentiation was confirmed using the neuron lineage-specific markers: mouse monoclonal anti-beta-tubulin isotype III (1:200, Sigma-Aldrich, Buchs, Switzerland) antibody and rabbit polyclonal anti-microtubule-associated protein 2 (anti-MAP2; 1:00, Sigma-Aldrich) antibody. The slides were incubated with the appropriate secondary antibodies, Cy3-conjugated goat anti-mouse and anti-rabbit secondary antibody (1:1000, Sigma-Aldrich), in the dark at room temperature for 1 h. The slides were counter-stained with DAPI (1:200, 4',6-diamidino-2-phenylindole, Sigma) and phalloidin (1:200, Sigma-Aldrich). The slides were analyzed using a Leica fluorescence microscope (CTR 6000).

The amount of cells expressing beta-tubulin isotype III or MAP-2 was determined by counting the dapi stained cells and comparing this to the number of cells staining positive for the two markers in at least three microscopic fields per each chamber slide. The percentage of cells expressing beta-III-tubulin/MAP2 in each experimental group and time point are presented in Fig. 3.

Western blotting

The cells were washed with cold PBS supplemented with a protease inhibitor cocktail (Sigma-Aldrich) and lysed with modified lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100 (Sigma), 2 mM EDTA, 10mM NapyroP, 200 uM Na3VO4, and 50 mM sodium fluoride). The protein lysate was measured using the BCA Protein Assay Kit (Thermo Scientific, Lausanne, Switzerland). 25 micrograms of the protein lysate of each sample was loaded on a 12% gel (Bio-Rad,

Cressier, Switzerland). The separated proteins were electro-transferred onto a PVDF membrane (Immobilon-P; Millipore, Bedford, MA). The primary antibodies were anti-beta-tubulin isotype III (1:500)), and anti-MAP2 (1:500). The membranes were washed and incubated with the appropriate HRP-conjugated secondary antibody (Amersham Pharmacia Biotech, Dübendorf, Switzerland) in TBS with 0.1% Tween-20 and 5% non-fat dry milk for 1 h. The signals on the membranes were detected through the ECL method (ECL-Kit, Amersham Pharmacia Biotech, Germany).

Statistical analysis

The data from cell proliferation, expression of beta-tubulin III and MAP-2 positive cells, and real-time PCR is presented as the mean ± SEM. In real-time PCR, two-way ANOVA with the Tukey test was used to determine the statistically significant increase in expression in stimulated cells compared to the control. A p-value of $p<0.05$ was used to define statistical significance.

Results

Copper electrolysis

Theoretical, calculated values for released copper due to applying the current of 1 mA or 1.5 mA were 24 µg/ml and 36 µg/ml, respectively. The weight of the working and counter electrodes was measured after applying the two currents. The measured mass of copper released with the current of 1 mA was 21 µg/ml and with the current of 1.5 mA 40 µg/ml. The difference between the theoretical and measured values for released copper was 12.5 % for the 1 mA and 10% for the 1.5 mA.

Cell viability and proliferation

Due to the stimulation, cell viability reduced to 78±11 %, measured right before and immediately after the 1 h stimulation. There was no significant difference in the cell viability between the different stimulation conditions. Cell proliferation was determined by counting dapi-stained cells in chamber slides at day 4, 7, and 14. Cells in all experimental conditions showed proliferation during the 14 days of culture (Fig. 2). By day 4, cells simulated with 1 mA alone or Cu + 1 mA had proliferated less than the control cells or cells stimulated with Cu + 1.5 mA or copper alone. At day 14, the cells stimulated with 1 mA or Cu + 1.5 mA showed no more proliferation whereas in other groups cells had continued to proliferate. However, none of the differences in proliferation between stimulation groups were significant ($p < 0.05$).

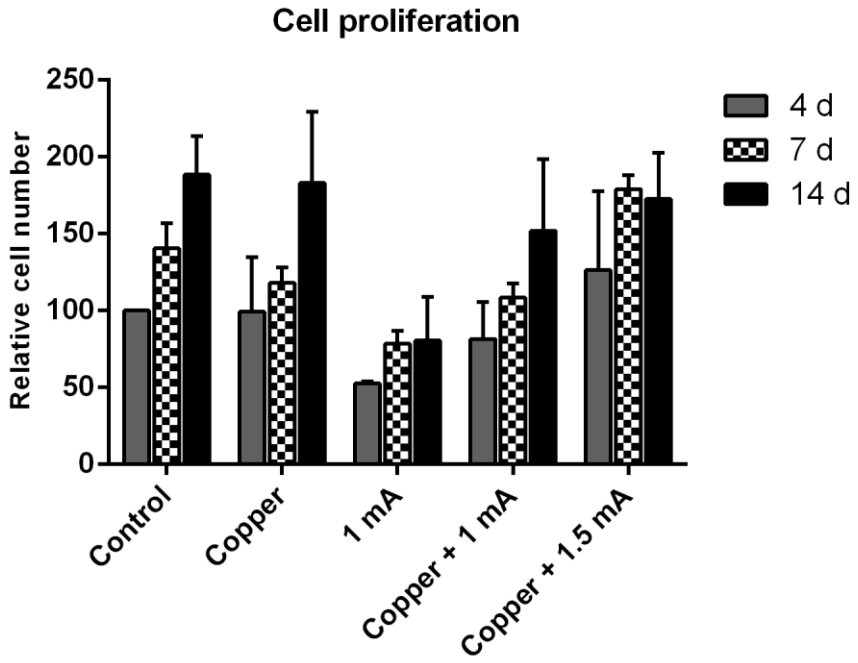


Figure 2. Cell proliferation during 14 days of culture. Cells from all the experimental groups showed proliferation during the culture time although the proliferation rates of cells stimulated with 1 mA alone and Cu + 1.5 mA were lower compared to control cells. The data represent the mean ± SEM of triplicates. There were no significant differences in proliferation between the experimental groups.

Cell morphology

To investigate the effect of the different stimulation conditions on the ADSCs, their morphological changes, which can be a sign of neuronal differentiation, were assessed by phalloidin staining and observed over time (figure 3). The exposure of the cells to an electric current resulted in a significant elongation of the cells compared to the control cells and cells stimulated with copper alone. Elongation was seen already at day 4 when the cells were exposed to current with or without the copper. Elongation was observed as a reduction of cytoplasm to nucleus ratio. Many cells showed neuron-like morphology with branches from the cell body (figure 3). These morphologies were maintained throughout the observation time of 14 days. Control cells and cells stimulated with copper alone maintained their adipose-like morphology and grew as a monolayer of large, flat cells that assume a spindle-shaped morphology at higher cell densities.

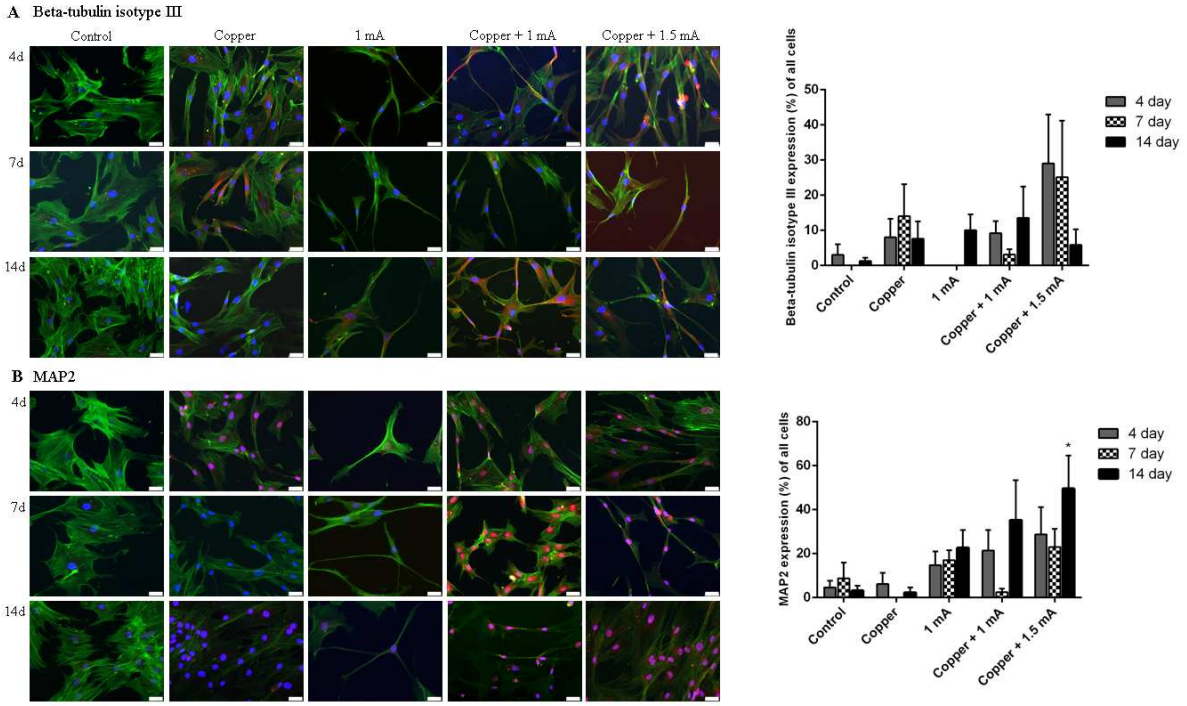


Figure 3. (A) Fluorescent images and graphs presenting the percentage of positive ADSC stained with anti-beta-tubulin isotype III neuronal antibody and (B) with anti-MAP2 neuronal antibody at 4, 7, and 14 days; anti-mouse or anti-rabbit CY3-conjugated secondary antibody (red), phalloidin (green), DAPI (blue). Scale bar is 50 μm. The data in graphs represent the mean ± SEM of triplicates. There were no significant differences in expression between the experimental groups apart from the MAP2 expression at day 14 when cells were stimulated with Cu + 1.5 mA.

Characterization of ADSC by immunohistochemistry

A positive expression of the beta-tubulin isotype III, a marker for immature neurons, was observed already at day 4 when the ADSCs were stimulated with copper with or without current, but the expression levels were clearly highest with the current of 1.5 mA and the copper gradually released to the cells via electrolysis (Cu + 1.5 mA). At day 7, the expression levels stayed approximately the same or decreased slightly. Cells stimulated with current alone remained negative at day 4 and 7. At day 14, only the ADSCs stimulated with both copper and current of 1 mA showed a further increase in expression of beta-tubulin isotype III whereas the expression in cells stimulated with copper alone or Cu + 1.5 mA decreased. Interestingly, a positive expression of cells stimulated with current only appeared only at day 14. There was no expression in control cells in any time point. The highest expression of beta-tubulin isotype III was observed at day 7 when cells were stimulated with Cu + 1.5 mA (Figure 3 A).

A positive expression of MAP-2, a mature neuronal marker, was observed in the cells stimulated with current alone or with both current and copper. Between day 4 and

day 14, the expression was increasing in these stimulation groups apart from the drop in expression in cells stimulated with Cu + 1 mA at day 7. Expression in all stimulation groups with current alone or both current and copper was highest at day 14 compared to the control. The highest expression was observed in cells stimulated with Cu + 1.5 mA. Control cells and cells stimulated with copper alone showed no MAP-2 expression in any time points. (Figure 3 B)

Shortly, only when ADSCs were stimulated with both copper and current (1 mA or 1.5 mA), there was a positive expression of both beta-tubulin isotype III and MAP-2. Also the highest expressions of both antibodies were detected when the stimulation combined both copper and current.

Characterization of neuron-like cells at the protein and mRNA levels

The western blot (WB) analysis showed increased levels of the neuronal protein beta-tubulin isotype III (46 kDa) in all of the experimental conditions at day 4 compared to the untreated control, except when the ADSCs were stimulated with current alone. The increase was highest when the ADSCs were stimulated with copper alone (figure 4). At day 7, the expression increased further within the cells stimulated with both current and copper. There was also a small increase in the expression in the cells stimulated with current only whereas in the copper alone stimulation, the expression had become almost negligible. At day 14, the beta-tubulin isotype III expression of copper + 1 mA had decreased to the same level as in the control and in other stimulation groups the expression was lower than in the control. The MAP-2 (239 kDa) protein expression at day 4 was higher in all of stimulation conditions compared to the untreated control except the current alone stimulation. At day 7, the expression had slightly decreased and there was no significant difference between the different stimulation conditions. At day 14, the expression had

decreased more, being almost at the same level with the control.

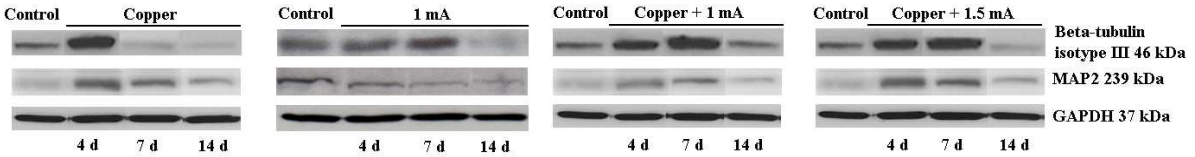


Figure 4. Neuron-specific protein expression of stimulated ADSCs. WB analysis showing the expression of (A) the 46-kDa beta-tubulin isotype III protein, and (B) the 239-kDa MAP-2 protein of the control cells and the stimulated cells at days 4, 7, and 14.

The stimulation of ADSCs with copper and current significantly increased the mRNA expression of beta-tubulin isotype III already at day 4 compared to the untreated control (figure 5A). Also cells stimulated with current alone showed a small, non-significant increase in beta-tubulin isotype III expression. Increase in beta-tubulin isotype III expression was greatest when the ADSCs were stimulated with 1 mA current and a gradual copper release via electrolysis (Cu + 1 mA). At day 7, expression levels of cells stimulated with both copper and current increased even more but then decreased by the day 14. The level stayed significantly higher than that of the control when the ADSCs were stimulated with both copper and 1 mA. When the cells were stimulated with copper or current alone, there was no significant increase in the expression levels except the 14 day expression of cell stimulated with 1 mA.

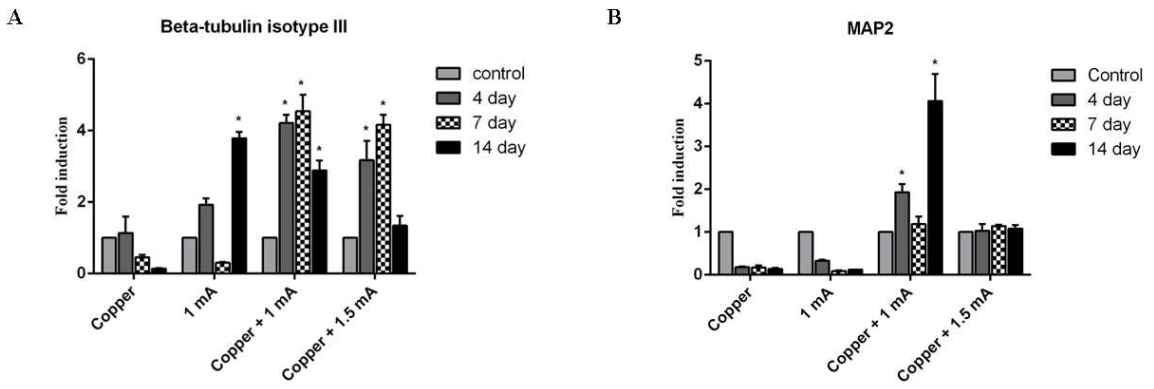


Figure 5. mRNA expression of neuron-specific markers in control ADSCs and stimulated ADSCs at days 4, 7, and 14. Induction of the gene expression of the neuronal markers (A) beta-tubulin isotype III and (B) MAP-2 was determined by real-time PCR using 18S as the control gene. The data represent the mean ± SEM of triplicates; the asterisk indicates $p < 0.05$ with respect to the control.

The MAP-2 mRNA levels were significantly increased compared to the control when the cells were stimulated with both copper and 1 mA. The highest expression was seen at day 14 whereas the other stimulation conditions did not show increase at any time points. (Fig. 5B).

6. Discussion

In this paper, we investigated if electric current and copper could be used to develop a growth factor-free method for the differentiation of ADSCs toward the neural lineage by a single application. In our preliminary studies, we saw neuron-like morphological changes in the ADSCs when they were exposed to current applied via electrodes containing copper, resulting in copper electrolysis in the medium. As we studied this further, in addition to changes in morphology, an elevated expression of neuronal markers was detected. When ADSCs were exposed to current alone using

pure platinum electrodes, some morphological changes characteristic to neurons were seen as well but there was a positive expression of only one neuronal marker and the expression level stayed lower compared to cells stimulated with both current and copper. The same applies to the cells stimulated with copper only.

We therefore hypothesized that the differentiation of ADSCs into neuron-like cells with a morphology characteristic to neurons and the expression of more than one neuronal marker requires the exposure of the cells to both electric current and copper. We investigated this effect in more detail and exposed the cells to two different currents and amounts of external copper. We studied the marker expression up to 14 days as it is commonly used latest time point in other studies[6,34] and allows enough time to detect the neuronal changes that can often be seen as early as 1-3 hours after induction[34].

Ariza et al. studied the stimulation of hippocampal neural progenitor cells with AC electric field of 46 mV/mm and continuous DC electric field of 437 mV/mm and were able to show that stimulation with 437 mV/mm, corresponding to a current of 3.38 mA, increased the neural differentiation tendency, whereas stimulation with 46 mV/mm had no significant influence on the cell alignment or on the cell differentiation [4]. However, in another study, hMSCs were differentiated towards neuron-like cells using a continuous DC electric field of 10 mV/mm [42]. In our study, we stimulated the cells with currents of 1 mA and 1.5 mA, corresponding to field strengths of 35 mV/mm and 53 mV/mm in Cu + 1 mA and Cu + 1.5 mA stimulations, and 155 mV/mm in 1 mA stimulation.

Stimulation of cells with an electric field causes changes in their morphology due to actin cytoskeleton reorganization and membrane-cytoskeleton dissociation [43]. It has been shown that ADSCs stimulated with a direct current elongate up to 145% of their

original length within 2 h [40]. We observed a similar and sustained elongation in the ADSCs exposed to current when compared to the untreated control and to cells stimulated with copper alone.

According to cell morphology and immunostaining, cells stimulated with copper showed some beta-tubulin isotype III expression, and cells stimulated with current had an elongated morphology. However, only cells stimulated with both copper and current showed both the morphological changes and beta-tubulin isotype III and MAP-2 expression. Similarly, the significant increase in beta-tubulin isotype III mRNA expression was observed when the cells were stimulated with both copper and current. At mRNA level the highest expression of beta-tubulin isotype III was detected at day 7 and in immunohistochemistry at protein level the highest expression was seen at day 14.

The only exception was the significant increase in beta-tubulin isotype III mRNA expression at day 14 when the cells were stimulated with current alone. Further time points beyond would be required to perceive if the high mRNA expression would be seen also as a high protein level expression after 14 days of culture. ADSCs stimulated with both copper and current, expressed the beta-tubulin isotype III mRNA as early as at day 4 after the stimulation and also protein expression could be seen in the earlier time points.

For beta-tubulin isotype III measured by WB, there was a higher expression in all stimulation conditions compared to control apart from the cells stimulated with current alone. The highest expression was seen in ADSCs stimulated with copper alone but this expression disappeared by day 7 whereas the expression remained high at day 7 when the ADSCs were stimulated with both copper and current that is also seen in the mRNA expression. After day 7, mRNA levels decreased and the decrease was seen also in beta-tubulin isotype III expression measured by WB.

Similarly to the beta-tubulin isotype III expression, the immunohistochemistry results showed that there was the strongest positive expression of MAP-2 when the ADSCs were stimulated with the combination of current and gradual copper release. In addition, immunostaining showed slightly elevated MAP-2 levels at day 4 also when ADSCs where stimulated with copper or current alone and this effect was also seen in WB. At the mRNA level, high MAP2 expression was observed only when the cells were stimulated with copper and 1 mA. Similarly, cells stimulated with copper and 1 mA showed increase in MAP-2 expression at day 4 and day 7 also in WB analysis; however, according to WB results, also stimulation with Cu + 1.5 mA increased the protein level expression at day 4 and day 7 which was not seen in real-time PCR.

Beta-tubulin isotype III is a characteristic marker for immature neurons. Immunohistochemical analyses have revealed high beta-tubulin isotype III expression levels in differentiating ADSCs after 14 days of neurogenic induction [6], which is in accordance with our immunostaining results. MAP-2 is expressed at the later time points by the mature neurons [1] and the high expression levels were maintained at day 14 only when cells were stimulated with both current and copper confirmed by immunostaining and real-time PCR.

The differences of the beta-tubulin isotype III and MAP-2 expression between the immunostaining and WB; namely immunostaining showing high expression at day 14 when the expression levels in WB had already decreased, could be explained by the cell proliferation; the relative amount of non-differentiated cells was increasing in the culture flask and as WB shows the total expression of protein, the expression levels were apparently decreased. Additionally, it is known that only a small proportion of ADSCs can differentiate into neurons [17] and therefore, by WB, lower expression of

neuronal markers was detected compared to the immunostaining where we looked at the neuron-positive cells.

In immunostaining after the stimulation, we saw no cytoplasmic changes nor damage to the nucleus that is usually a sign of the lack of cell stress or death [23,29]. In addition, cells were proliferating in the chamber slides and we detected no significant cell death due to the copper or current after the cells had been seeded to the chamber slides or culture flasks after the stimulation.

Our results indicate that the differentiation of ADSCs toward the neuronal lineages can be triggered by using combination of an electric current and continued copper stimulation. Electric fields are known to be related to the neuronal differentiation of embryonic, neural and mesenchymal stem cells [4,25,42,45]. It is possible that the differentiation process can take place also when stimulating cells with electric field only but the upregulation of beta-tubulin isotype III, a marker for immature neurons, was seen only after 14 days of culture, compared to 4 days when using electric field and copper together. The mature neuronal marker, MAP-2, showed no increase in expression levels within the 14 days of culture when the ADSCs were stimulated with current alone. Copper is well known to play an important role in brain development [24,28]. High amounts of copper are also stored in the synaptic terminals of central neurons [36], and it has been shown that copper can modify neuronal excitability [13].

Rodrigues et al. showed that an increase in the extracellular concentration of copper modifies the proliferation and differentiation of mesenchymal stem cells (MSCs) *in vitro*. Their results show that the addition of copper to a culture of MSCs in osteogenic or adipogenic differentiation media decreased the proliferation and promoted the osteogenic or adipogenic differentiation of MSCs, respectively. The authors concluded that copper plays a role either in the commitment of the MSCs

toward a specific differentiation pathway or in the maturation of the chosen cell lineage [32]. Monson et al. (2012) showed that phosphatidylserine (PS) binds Cu^{2+} with very high affinity. PS is a phospholipid that is found in the cell membrane and is particularly enriched in neuronal membranes [19]. Thus, the stimulation of ADSCs with copper may result in Cu^{2+} binding to the PS in the cell membrane to initiate the commitment step in the differentiation toward the neuronal lineage.

According to our results, stimulating ADSCs with both copper and current seems to induce the highest and fastest expression of the two neuronal markers. Here used current densities and consequent copper concentrations are high enough to induce the neuron-like differentiation of ADSCs but we did not observe clear causality between the two current densities. We hypothesize that external electric field is enabling a more efficient intake of copper into the cell. This may be due to the reassembly or redistribution of the phospholipid PS, or generally phospholipids by electric field, reported in several studies[30,47]. Electric stimulation can also result in displacement of membrane proteins[30], such as copper transporter Ctr1 and thus influence the copper intake.

Generally, when stimulating cells or tissues with electrodes, also the generation of reactive oxygen species (ROS) occurs. High amounts of ROS can cause cell death and disrupt or inhibit gene expression but at lower levels ROS can increase the proliferation and differentiation by activating many signaling cascades and transcription factors [18,37–39]. However, the actual effect of ROS in this stimulation setup still needs to be investigated.

In conclusion, stimulation with electric fields combined with a sustained release of copper could provide a feasible, non-expensive, growth factor-free method for the

differentiation of ADSCs toward the neuronal lineage indicated by morphological changes and upregulation of neuron-specific genes and proteins. However, differentiation of functional neurons is a complicated process and neuron-like morphology and expression of neuronal markers beta-tubulin isotype III and MAP-2 alone does not prove that the ADSCs become functional neurons. Therefore, in this study we have shown that stimulation with electric currents and copper induces neuron-like changes in the cell morphology as well as gene and protein expression but in the future, the neuron functionality has to be verified in order to confirm that the ADSCs can differentiate into mature neurons.

Our in vitro experiments offer information regarding growth factor-free differentiation of ADSC to neuron-like cells which can be a base for further pre-clinical studies. Further animal studies are required to evaluate the tissue response, nerve regeneration and functionality of these neuron-like cells in vivo.

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8. References

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